SPECIFICATION

A Gene Coding for Penicillin Binding Protein and a Method for Producing L-Glutamic Acid

Technical Field

The present invention relates to a method for producing L-glutamic acid by fermentation. L-glutamic acid is widely used as a raw material of seasonings and so forth.

Background Art

If coryneform bacteria are cultured in a medium containing a restricted amount of biotin, the bacteria produce a marked amount of L-glutamic acid. On the other hand, if coryneform bacteria are cultured in a medium containing an excessive amount of biotin, the bacteria do not produce L-glutamic acid. However, it is known that, even under such a condition, if a surface active agent or a biotin activity suppressor such as penicillin is added to the medium, growth of the bacteria is suppressed and they become to produce a marked amount of L-glutamic acid.

The glutamic acid production induced by addition of penicillin to a medium has been studied for many

years (T.D. Nunheimer, J. Birnbaum, E.D. Ihnen and A.L. Demasin, *Appl. Microbiol.*, *20*, 215-217 (1970)). The effect of penicillin is considered to cause structural change of cellular surface layers, thereby enhancing permeability of cytoplasmic membranes for glutamic acid.

In addition, it has also been studied through what kind of mechanism the restriction of the biotin amount or the addition of a surface active agent or penicillin influences the productivity of L-glutamic acid of coryneform bacteria, and presence of a gene considered to participate in the L-glutamic acid production has been elucidated (*dts*R gene). Further, it has been confirmed that a strain of which *dts*R gene is disrupted produces a marked amount of L-glutamic acid even under a condition in which biotin is present in such an amount that a wild strain hardly produces L-glutamic acid (International Patent Publication WO95/23224).

Furthermore, there have been obtained many findings that contradicts the explanation that the glutamic acid production is induced the enhancement of permeability of cytoplasmic membranes, and the mechanism of the glutamic acid production induced by penicillin has still been unknown (E. Kimura, Y. Kawahara and W. Nakamatsu, *Tanpakusshitu Kakusan Koso* (*Protein, Nucleic acid and Enzyme*), vol. *42*, pp.2633-2640 (1997)).

By the way, it is well known that penicillin binding proteins (PBPs) play an important role in

bacterial cell division. The penicillin binding proteins are considered to be enzymes that exist in bacterial cellular surface layers, and they specifically bind to β-lactam antibiotics such as penicillin. Although it may vary depending on the species, it is considered that 3-8 kinds of the proteins are usually found in *Escherichia coli*, and their molecular weights are distributed around the range of 40,000-120,000. Penicillin inhibits the enzymatic reactions by binding to a serine residue of an active site of the penicillin binding proteins.

It has been elucidated that 7 kinds of penicillin binding proteins exist in *Escherichia coli* (*E. coli*), which was especially a main target of researches (B.G. Spratt, *Proc. Natl. Acad. Sci. U.S.A.*, *72*, 2999 (1975)). Among those, those designated as PBP2 and PBP3 have been demonstrated to play an important role in cell division (B.G. Spratt, *J. Bacteriol.*, *131*, 293 (1977)). However, it has not been known whether penicillin binding proteins exists in coryneform bacteria.

Disclosure of the Invention

The inventors of the present invention investigated whether penicillin binding proteins (abbreviates as "PBPs" hereinafter) should exist also in coryneform bacteria, and analyzed their functions. As a

result, the inventors obtained a novel finding considered to be useful for elucidating the mechanism of the glutamic acid production in coryneform bacteria induced by penicillin, and at the same time, they found that the finding could be utilized for developments concerning the improvement of glutamic acid producing ability of coryneform bacteria from a viewpoint that had not been known yet.

The present invention was accomplished on the basis of the aforementioned findings, and it relates to a method for producing L-glutamic acid, comprising the steps of cultivating a coryneform bacterium in a liquid medium to produce and accumulate L-glutamic acid in the medium, and collecting the L-glutamic acid, wherein PBP does not normally function in the bacterium and the bacterium has the ability to produce L-glutamic acid

In a preferred embodiment of the present invention, the coryneform bacterium used in the aforementioned method is a bacterium in which PBP functions normally at the first temperature and does not function normally at the second temperature, and the method comprises a step of cultivating the bacterium at the first temperature to proliferate the bacterium and a step of cultivating the bacterium at the second temperature to produce L-glutamic acid.

In another embodiment of the present invention, the coryneform bacterium used in the aforementioned

method is a bacterium which harbors a plasmid containing a gene coding for PBP (PBP gene) and a temperature sensitive replication control region, and in which the PBP gene on chromosome does not function, and the plasmid can replicate at the first temperature, and cannot replicate at the second temperature.

In a further embodiment of the present invention, PBP produced by the coryneform bacterium used in the method has a temperature sensitive mutation.

In a still further embodiment of the present invention, PBP shows a molecular weight of about 60,000 in SDS-polyacrylamide gel electrophoresis, when PBP binds to penicillin G.

The second aspect of the present invention is DNA which codes for a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 2 in Sequence Listing;

(B) a protein which has an amino acid sequence of SEQ ID NO: 2 in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and an activity for binding to penicillin.

In a preferred embodiment of the invention, the aforementioned DNA is DNA defined in the following (a) or (b):

(a) DNA which comprises at least the nucleotide

sequence of the nucleotide numbers 881 to 2623 of SEQ ID NO: 1 in Sequence Listing;

(b) DNA which is hybridizable with a nucleotide sequence comprising at least the sequence of the nucleotide numbers 881 to 2623 of SEQ ID NO: 1 in Sequence Listing under a stringent condition, and codes for a protein having an activity for binding to penicillin.

Hereafter, the present invention will be explained in detail.

<1> PBP of coryneform bacteria

In the present invention, coryneform bacteria include those bacteria having been hitherto classified into the genus *Brevibacterium* but united into the genus *Corynebacterium* at present (*Int. J. Syst. Bacteriol., 41,* 255 (1981)), and include bacteria belonging to the genus *Brevibacterium* closely relative to the genus *Corynebacterium*. Examples of such coryneform bacteria are listed below.

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium callunae

Corynebacterium glutamicum

Corynebacterium lilium (*Corynebacterium glutamicum*)

Corynebacterium melassecola

Brevibacterium divaricatum

(*Corynebacterium glutamicum*)

Brevibacterium flavum (*Corynebacterium glutamicum*)

Brevibacterium immariophilum

Brevibacterium lactofermentum

(*Corynebacterium glutamicum*)

Brevibacterium roseum

Brevibacterium saccharolyticum

Brevibacterium thiogenitalis

Corynebacterium thermoaminogenes

Specifically, the following strains can be exemplified.

Corynebacterium acetoacidophilum ATCC 13870

Corynebacterium acetoglutamicum ATCC 15806

Corynebacterium callunae ATCC 15991

Corynebacterium glutamicum ATCC 13020

Corynebacterium lilium (*Corynebacterium glutamicum*) ATCC 15990

Corynebacterium melassecola ATCC 17965

Brevibacterium divaricatum

(*Corynebacterium glutamicum*) ATCC 14020

Brevibacterium flavum (*Corynebacterium glutamicum*) ATCC 14067

Brevibacterium immariophilum ATCC 14068

Brevibacterium lactofermentum

(*Corynebacterium glutamicum*) ATCC 13869

Brevibacterium roseum ATCC 13825

Brevibacterium saccharolyticum ATCC 14066

Brevibacterium thiogenitalis ATCC 19240

Corynebacterium thermoaminogenes AJ12340

(FERM BP-1539)

PBP referred to in the present invention is a membrane protein that exists in the cellular surface layers of the above coryneform bacteria, and if it contacts with penicillin, it binds thereto with a covalent bond. PBP can be detected by, for example, adding labeled penicillin to a membrane fraction of coryneform bacteria to allow a reaction, subjecting a surface active agent-soluble fraction to electrophoresis on polyacrylamide gel, and visualizing the label (penicillin binding test). As shown in the examples mentioned below, PBPs of *Brevibacterium lactofermentum* are detected as three bands at molecular weights of about 110 kDa, 100 kDa and 60 kDa in SDS polyacrylamide gel electrophoresis (SDS-PAGE) in a state that they bind to penicillin G, and they were designated as PBP1, PBP2 and PBP3.

When affinity between these PBPs and mecillinam, which is a derivative of penicillin G, was investigated, it was found that mecillinam selectively binds to PBP3. Further, it was found that, when *Brevibacterium lactofermentum* was cultured under a condition that

biotin was present in a significant amount, L-glutamic acid was produced in the presence of a certain concentration of mecillinam. These facts suggested that L-glutamic acid production could be induced in the presence of a significant amount of biotin by inhibiting the function of PBPs, at least PBP3. Therefore, a coryneform bacterium in which PBP does not normally function should come to be able to induce L-glutamic acid production without addition of a biotin activity suppressor even in the presence of a significant amount of biotin. Moreover, there is possibility that such a coryneform bacterium in which PBP does not normally function should have improved L-glutamic acid producing ability. Furthermore, a novel finding concerning the L-glutamic acid production of coryneform bacteria should be obtained by manipulating a PBP gene, and it can be utilized for development from an aspect that have not been known yet.

In the present invention, the expression "PBP does not normally function" means a state that the L-glutamic acid production is induced in the presence of a significant amount of biotin. It may be a state that PBP is not produced because of inhibition of transcription or translation of a PBP gene, or a state that the function of PBP is reduced or eliminated because of a mutation occurring in the produced PBP.

<2> Coryneform bacterium in which PBP does not function normally

The coryneform bacterium used for the method for producing of L-glutamic acid according to the present invention is a coryneform bacterium in which PBP does not function normally. In a preferred embodiment, the coryneform bacterium used in the aforementioned method is a bacterium in which PBP functions normally under the first culture condition and does not function normally under the second culture condition. Such a coryneform bacterium can proliferate under the first culture condition, and can produce L-glutamic acid under the second culture condition in the presence of a significant amount of biotin. As PBP, PBP3 is preferred.

As the aforementioned culture condition, there can be mentioned temperature of culture, osmotic pressure of medium, pH, components of medium and so forth. Examples of the components of medium include an inducer such as IPTG (isopropyl-β-D-thiogalactopranoside) and acetic acid, and a suppressor such as glucose. The culture temperature will be explained as an example of the culture condition in the description hereinafter. However, concerning other culture conditions, the term "temperature" in the following description can be replaced with a term indicating another condition.

As an example of the coryneform bacterium in which PBP does not function normally, there can be mentioned a

mutant strain in which a mutation is introduced into a gene coding for PBP (PBP gene) so that PBP that functions normally should not be expressed. The aforementioned mutation may be a mutation that inhibits transcription or translation of the PBP gene, or a mutation that produces PBP that does not function normally.

Since a mutation that causes complete deficiency of PBP should be fatal to the bacteria, the aforementioned mutant strain can be obtained as a conditional mutant strain such as a temperature sensitive mutant strain. Such a mutant strain can be obtained by, for example, treating a coryneform bacterium by ultraviolet irradiation or with a chemical agent to obtain mutant strains that can proliferate at the first temperature (for example, low temperature) but cannot proliferate at the second temperature (for example, high temperature), and selecting, from the obtained mutant strains, a mutant strain that can proliferate at the first temperature and produces L-glutamic acid in the presence of a significant amount of biotin when it is cultured at the second temperature. As a mutant strain that represents such a characteristic, a DTSR protein deficient strain (International Patent Publication WO95/23224) or an α-KGDH deficient strain (International Patent Publication WO95/34672) may be selected. However, it can be confirmed if a candidate

strain is a mutant strain having a mutation concerning PBP by performing the aforementioned penicillin binding test for the cells cultured at the second temperature.

If such a mutant strain is once obtained, a PBP gene of the coryneform bacterium can be cloned by using the mutant strain as a host. That is, a DNA fragment containing a PBP gene can be obtained by transforming the mutant strain in which PBP does not function normally with a plasmid containing chromosomal DNA derived from coryneform bacteria, selecting a transformant strain in which PBP functions normally, and collecting the plasmid.

Techniques used for usual gene recombination such as those for digestion and ligation of DNA, transformation, extraction of recombinant DNA from transformants, colony hybridization and so forth are described in references well known to those skilled in the art, for example, J. Sambrook, E.F. Fritsch, and T. Maniatis, Molecular Cloning, Cold Spring Harbor Laboratory Press (1989) and so forth.

A chromosomal DNA library can be produced, for example, as follows. First, chromosomal DNA is prepared by the method of Miura et al. (H. Saito, and K. Miura, Biochem. Biophys. Acta, 72, 619 (1963)) or the like. Then, the chromosomal DNA is partially digested with a suitable restriction enzyme to obtain a mixture of various fragments. If the degree of the digestion is

controlled by adjusting digestion reaction time or the like, restriction enzymes of a wide range can be used. For example, the chromosomal DNA is digested by allowing *Sau*3AI to act on it at a temperature of 30°C or higher, preferably 37°C or higher, for various periods of time (1 minute to 2 hours) at an enzyme concentration of 1-10 units/ml.

Subsequently, the digested chromosomal DNA fragments are ligated to vector DNA autonomously replicable within *Escherichia coli* cells to produce recombinant DNA. More specifically, a restriction enzyme producing the same end nucleotide sequence as the restriction enzyme used for the digestion of the chromosomal DNA, *Sau*3AI, for example, *Bam*HI, is allowed to act on the vector DNA at a temperature of 30°C or higher for 1 hour or more, preferably 1-3 hours, at an enzyme concentration of 1-100 units/ml to fully digest the vector and cleave it. Then, the mixture of the chromosomal DNA fragments and the cleaved vector DNA obtained as described above are mixed, and a DNA ligase, preferably T4 DNA ligase, is allowed to act on the mixture at a temperature of 4-16°C for 1 hour or more, preferably 6-24 hours, at an enzyme concentration of 1-100 units/ml to obtain recombinant DNA.

The vector autonomously replicable in *Escherichia coli* cells is preferably a plasmid vector autonomously replicable in the host cell, and examples thereof

include pUC19, pUC18, pBR322, pHSG299, pHSG399, pHSG398, RSF1010 and so forth.

Moreover, if a DNA fragment having an ability to make a plasmid autonomously replicable in coryneform bacteria (it can be prepared from, for example, pAM330 (refer to Japanese Patent Laid-open (Kokai) No. 58-67699), pHM1519 (refer to Japanese Patent Laid-open No. 58-77895), pCG1 (refer to Japanese Patent Laid-open No. 57-134500), pCG2 (refer to Japanese Patent Laid-open No. 58-35197), pCG4 (refer to Japanese Patent Laid-open No. 57-183799), pCG11 (refer to Japanese Patent Laid-open No. 57-183799) and so forth) is inserted into the aforementioned vectors, they can be used as a so-called shuttle vector autonomously replicable in both of *Escherichia coli* and coryneform bacteria.

Examples of such a shuttle vector include those mentioned below. There are also indicated microorganisms that harbor each vector, and accession numbers thereof at international depositories are shown in the parentheses, respectively.

pAJ655: *Escherichia coli* AJ11882 (FERM BP-136)
Corynebacterium glutamicum SR8201 (ATCC 39135)
pAJ1844: *Escherichia coli* AJ11883(FERM BP-137)
Corynebacterium glutamicum SR8202 (ATCC39136)
pAJ611: *Escherichia coli* AJ11884 (FERM BP-138)
pAJ3148: *Corynebacterium glutamicum* SR8203 (ATCC 39137)

pAJ440: *Bacillus subtilis* AJ11901 (FERM BP-140)

By using the obtained recombinant DNA, for example, *Escherichia coli* K-12 strain is transformed to prepare a chromosomal DNA library. This transformation can be performed by the method of D.M. Morrison (*Methods in Enzymology*, *68*, 326, 1979), the method comprising treatment of recipient cells with calcium chloride to increase their permeability for DNA (M. Mandel, and A. Higa, *J. Mol., Biol., 53*, 159 (1970)) or the like.

Then, a mutant strain of a coryneform bacterium in which PBP does not function normally is transformed with the obtained chromosomal DNA library. As the method for transformation of coryneform bacteria, there is the aforementioned method utilizing a treatment of cells with calcium chloride or a method comprising allowing cells at a particular growth phase where they can take up DNA to take up it (reported for *Bacillus subtilis* by C.H. Duncan et al.). In addition to these, also employable is a method of making DNA-recipient cells into protoplasts or spheroplasts which can easily take up recombinant DNA, followed by allowing introduction of the recombinant DNA into the cells, which is known to be applicable to *Bacillus subtilis*, actinomycetes and yeasts (S. Chang, *et al., Molec. Gen. Genet., 168*, 111 (1979); Bibb, *et al., Nature, 274*, 398 (1978); A. Hinnen, *et al., Proc. Natl. Sci. USA, 75*, 1929 (1978)). Other

than those, a method for transformation of coryneform bacteria is also disclosed in Japanese Patent Laid-open No. 2-207791).

A transformant strain introduced with a PBP gene can be obtained by plating the transformed mutant strain under a condition where a mutant strain (host) that is not transformed cannot grow, and selecting a strain that has formed a colony. A DNA fragment containing a PBP gene can be obtained from the obtained transformant by isolating the recombinant DNA, for example, by the method of P. Guerry, *et al.* (*J. Bacteriol.*, *116*, 1064 (1973)), the method of D.B. Clewell (*J. Bacteriol.*, *110*, 667 (1972)) or the like. A coryneform bacterium transformed with recombinant DNA containing a PBP gene can also possibly be obtained by hybridization using a gene coding for a known PBP of a microorganism, for example, PBP of *Escherichia coli*, or an oligonucleotide produced based on the nucleotide sequence thereof.

Besides the mutagenesis treatment, a coryneform bacterium in which PBP does not function normally can be created by using a PBP gene obtained as described above. A PBP gene on chromosome can be disrupted by transforming a coryneform bacterium with DNA containing a PBP gene modified with internal deletion so as not to produce PBP functioning normally (deletion type PBP gene), and allowing recombination between the deletion type PBP gene and the PBP gene on the chromosome. Such

gene destruction by homologous recombination has already been established, and there are methods utilizing a linear DNA, a plasmid that contains a temperature sensitive replication control region and so forth. In the present invention, the method utilizing a plasmid that contains a temperature sensitive replication control region is preferred.

A PBP gene on host chromosome can be replaced with the deletion type PBP gene as follows. That is, recombinant DNA is first prepared by inserting a temperature sensitive replication control region, mutant PBP gene and marker gene for resistance to a drug such as chloramphenicol, with which recombinant DNA a coryneform bacterium is transformed. Further, the resultant transformant strain is cultured at a temperature at which the temperature sensitive replication control region does not function, and then the transformant strain can be cultured in a medium containing the drug to obtain a transformant strain in which the recombinant DNA is incorporated into the chromosomal DNA.

In such a strain in which recombinant DNA is incorporated into chromosomal DNA, the mutant PBP gene is recombined with the PBP gene originally present on the chromosome, and the two fusion genes of the chromosomal PBP gene and the deletion type PBP gene are inserted into the chromosome so that the other portions

of the recombinant DNA (vector segment, temperature sensitive replication control region and drug resistance marker) should be present between the two fusion genes. Therefore, the transformant expresses PBP, because the normal PBP gene is dominant in this state, and it can grow.

Then, in order to leave only the deletion type PBP gene on the chromosomal DNA, one copy of the PBP gene is eliminated together with the vector segment (including the temperature sensitive replication control region and the drug resistance marker) from the chromosomal DNA by recombination of the two PBP genes. In that case, the normal PBP gene is left on the chromosomal DNA, and the deletion type PBP gene is excised from the chromosomal DNA, or to the contrary, the deletion type PBP gene is left on the chromosomal DNA, and the normal PBP gene is excised from the chromosome DNA. In the both cases, the excised DNA may be retained in the cell as a plasmid when the cell is cultured at a temperature at which the temperature sensitive replication control region can function. Subsequently, the cell is cultured at a temperature at which the temperature sensitive replication control region cannot function. In the culture, the cell cannot proliferate when the deletion type PBP gene is left on the chromosomal DNA, since the plasmid containing the normal PBP gene is dropped from the cell. On the other hand, when the normal PBP gene

is left on the chromosomal DNA, the cell can proliferate. Thus, a strain in which the PBP gene on the chromosomal DNA is disrupted and the normal PBP gene is harbored on the plasmid can be obtained by selecting a strain that can proliferate at a temperature at which the temperature sensitive replication control region functions, but cannot proliferate at a temperature at which the temperature sensitive replication control region does not function.

When the strain with the disrupted PBP gene obtained as described above is cultured at a temperature at which the temperature sensitive replication control region functions (for example, low temperature), it retains the PBP gene in the cell, and when it is cultured at a temperature at which the temperature sensitive replication control region does not function (for example, high temperature), it loses the PBP gene. In the following description, a temperature at which a temperature sensitive plasmid cannot replicate is referred to as a high temperature. However, it is not intended to exclude possibility that the temperature is a low temperature, and if a temperature sensitive replication control region is obtained which cannot replicate at a low temperature but can replicate at a high temperature, it may also be used.

A strain which is made recA$^-$ after the PBP gene on chromosome DNA is disrupted and the normal PBP gene is

incorporated into a plasmid is preferably used as the microorganism used in the present invention, since in such a strain, incorporation of the PBP gene on the plasmid into the chromosome during culture at a low temperature is prevented and thus the drop of the gene is secured.

The temperature sensitive replication control region can be obtained by subjecting a plasmid autonomously replicable in a coryneform bacterium cell and having drug resistance to a mutagenesis treatment, transforming a coryneform bacterium with the plasmid and extracting a plasmid from a transformant that can grow at a low temperature but cannot grow at a high temperature in a medium containing the drug.

As the method for the mutagenesis of plasmid, there can be mentioned the method utilizing an in vitro treatment of plasmid with hydroxylamine (G.O. Humpherys, *et al., Molec. Gen. Genet., 145,* 101-108 (1976) etc.).

The terms "low temperature" and "high temperature" herein used have relative concepts, and the border between the low temperature and the high temperature is not particularly limited. The "low temperature" means a temperature range wherein coryneform bacteria can at least proliferate when they are cultured. The "high temperature" means a temperature wherein coryneform bacteria themselves shall not die. The border of the low temperature and the high temperature can be

determined by cultivating a transformant harboring a temperature sensitive plasmid in a medium containing a drug at various temperatures to determine a lower limit temperature above which the transformant cannot grow.

Examples of the plasmid that has a temperature sensitive replication control region functioning in a coryneform bacterium cell include pHS4, pHS22 and pHS23. Plasmid pHSC4, which is obtained by ligating a DNA fragment excised from pHS4 and containing a replication control region derived from a coryneform bacterium to a vector for *Escherichia coli*, pHSG398, can also be used for the present invention as a temperature sensitive plasmid. pHSC4 autonomously proliferates in coryneform bacteria and *Escherichia coli*, and it imparts chloramphenicol resistance to a host. *Escherichia coli* AJ12571 harboring pHSC4 was deposited at the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology, the Ministry of International Trade and Industry on October 11, 1990, and received an accession number of FERM P-11763. Then, it was transferred to an international deposit under the provisions of the Budapest Treaty on August 26, 1991, and received an accession number of FERM BP-3524.

These temperature sensitive plasmids can autonomously proliferate at about 10-32°C in a coryneform bacterium cell, but they cannot autonomously proliferate at about 34°C or higher.

A DNA fragment that has a temperature sensitive replication control region can be obtained by, for example, digesting the aforementioned pHSC4 with *Bam*HI and *Kpn*I.

Construction of the aforementioned plasmids and nucleotide sequences of the regions containing a temperature sensitive replication control region thereof are disclosed in Japanese Patent Publication (Kokoku) No. 7-108228.

It is also known that, in *Escherichia coli*, some of a penicillin binding protein genes form a gene cluster, and murE gene and PBP3 gene (ftsI) are located closely to each other (F. Ishino, Nippon Nogeikagaku Kaishi, vol.63, No.11, 1755-1764 (1989)). Therefore, a PBP gene may be obtained together with the murE gene by transforming a murE temperature sensitive mutant as a host, which has been already obtained for coryneform bacteria, with a plasmid containing chromosomal DNA derived from a coryneform bacterium to obtain a transformant strain that has come to be able to grow at a temperature at which the host cannot grow. The inventors of the present invention successfully obtained a PBP gene based on this concept as described in the examples mentioned below.

Further, since the nucleotide sequences of the PBP gene and flanking regions were elucidated by the present invention, the PBP gene can readily be obtained by

preparing primers based on the sequence and performing PCR (polymerase chain reaction; refer to T.J. White, *et al.*, *Trends Genet.*, *5*, 185 (1989)) utilizing coryneform bacterium chromosomal DNA as a template.

The nucleotide sequence containing the PBP gene obtained in the examples mentioned later is shown as SEQ ID NO: 1 in Sequence Listing. In this nucleotide sequence, there exist three opening reading frames (ORF) (nucleotide numbers 881-2623, 2790-4454 and 4467-5345). The amino acid sequences encoded by the ORFs are shown as SEQ ID NOS: 2-4.

When an existing protein database was searched for sequences exhibiting homology with these amino acid sequences, it was found that the amino acid sequence encoded by the first OFR showed homology of about 31% with the amino acid sequence encoded by the PBP3 gene (*ftsI*) of *Escherichia coli* on the amino acid level. The amino acid sequences encoded by the second and the third OFRs showed homology to the amino acid sequences encoded by the murE gene and the murF gene of *Escherichia coli*, respectively. These results suggested that the first OFR is the PBP gene corresponding to the *ftsI* of *Escherichia coli*.

The PBP gene of the present invention may code for substitution, deletion, insertion, addition or inversion of one or several amino acids, so long as the activity of the encoded protein for binding to penicillin is not

degraded. The number meant by the term "several" used herein may vary depending on locations in the three-dimensional structure of proteins and kinds of amino acid residues. This is due to the fact that there are analogous amino acids among amino acids such as isoleucine and valine, and difference among such amino acids does not substantially affect the three-dimensional structure of proteins. Therefore, the encoded protein may be one having homology of 30-40% or more, preferably 55-65% or more, with respect to the entire amino acid sequence constituting the protein, and having the activity for binding to penicillin.

Such DNA encoding a protein substantially the same as a penicillin binding protein as mentioned above can be obtained by modifying the nucleotide sequence by, for example, the site-directed mutagenesis so that the amino acid residues of a specific site should include substitution, deletion, insertion, addition or inversion. Such modified DNA as mentioned above can also be obtained by an already known mutagenesis treatment. Examples of the mutagenesis treatment include in vitro treatment of DNA coding for a penicillin binding protein with hydroxylamine etc., treatment of a microorganism having DNA coding for a penicillin binding protein, for example, *Escherichia* bacteria, by ultraviolet irradiation or with a mutagenizing agent used for usual mutagenesis treatment such as N-methyl-N'-nitro-N-

nitrosoguanidine (NTG) or nitrous acid.

The substitution, deletion, insertion, addition or inversion of nucleotides described above also includes mutation (mutant or variant) that naturally occurs due to individual difference, difference in species or genera of the microorganism having a penicillin binding protein.

DNA coding for substantially the same protein as a penicillin binding protein can be obtained by expressing DNA having such a mutation as described above in an appropriate cell, and examining the penicillin binding activity of an expressed product. DNA coding for substantially the same protein as a penicillin binding protein can also be obtained by isolating DNA hybridizable with DNA having, for example, the nucleotide sequence of nucleotide numbers 881 to 2623 in SEQ ID NO: 1 in Sequence Listing under a stringent condition and coding for a protein having the penicillin binding activity from DNA coding for penicillin binding protein having mutation or from a cell harboring it. The "stringent condition" referred to herein is a condition under which a so-called specific hybrid is formed, and a non-specific hybrid is not formed. It is difficult to clearly express this condition by using numerical values. However, for example, the stringent condition includes a condition under which two of DNA having high homology, for example, two of DNA having

homology of not less than 50% are hybridized with each other, and two of DNA having homology lower than the above are not hybridized with each other. Alternatively, the stringent condition is exemplified by a condition under which two of DNA are hybridized with each other at a salt concentration corresponding to an ordinary condition of washing in Southern hybridization, i.e., 60°C, 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS.

The genes hybridizable under the condition as described above includes those having a stop codon generated in the gene, and those having no activity due to mutation of the active center. However, such mutant genes can be easily removed by ligating the genes with a commercially available activity expression vector, and measuring the penicillin binding activity.

<3> Production of L-glutamic acid

L-Glutamic acid can be produced by cultivating such a coryneform bacterium as described above in which PBP does not function normally and which has L-glutamic acid producing ability in a liquid medium to produce and accumulate L-glutamic acid in the medium, and collecting the L-glutamic acid. According to the method of the present invention, L-glutamic acid can be produced in a medium containing a significant amount of biotin such as molasses without adding a biotin activity inhibitor.

When the coryneform bacterium used is a bacterium

of which PBP normally functions at the first temperature and does not normally function at the second temperature, the bacterium is cultured at the first temperature to allow it to proliferate, and then cultured with shifting the temperature to the second temperature to produce L-glutamic acid.

Specifically, the temperature shifting can be attained by, for example, performing seed culture at a low temperature and performing culture in a main medium (main culture) at a high temperature. The temperature may also be shifted during preculture or main culture. The step of cell proliferation and the step of drop of plasmid are not clearly distinguished, and the step of drop of plasmid is accompanied with proliferation of cells.

As for the timing of the temperature shift, the period for the culture at a low temperature can readily be decided by carrying out preliminarily experiments with various periods of culture until the temperature shift. In general, the culture can be continued until a desired cell density is attained in the logarithmic growth phase, and then the temperature can be shifted to a level at which the plasmid cannot replicate.

The medium used for the culture is not particularly limited, and there can be used a usual medium containing a carbon source, nitrogen source, and inorganic ions as well as organic trace nutrients as

required. In the present invention, in particular, a medium containing a significant amount of biotin may be used.

As the carbon source, there can be used saccharides such as glucose, lactose, galactose, fructose and hydrolysate of starch, alcohols such as ethanol and inositol, organic acids such as acetic acid, fumaric acid, citric acid and succinic acid and so forth.

As the nitrogen source, inorganic ammonium salts such as ammonium sulfate, ammonium chloride and ammonium phosphate, organic nitrogen such as soybean hydrolysate, ammonia gas, aqueous ammonia and so forth can be used.

As the inorganic ions or sources thereof, a small amount of potassium phosphate, magnesium sulfate, iron ions, manganese ions and so forth may be added. As a trace amount organic nutrient, it is desirable to add a suitable amount of required substances such as vitamin B_1 and yeast extract and so forth as required.

The culture is preferably performed for 16 to 72 hours under an aerobic condition, and the culture temperature is controlled to be 20 to 45°C, and pH to be 5-8.5 during the culture. For adjusting pH, inorganic or organic acidic or alkaline substances, ammonia gas and so forth can be used.

Collection of L-glutamic acid from the culture can usually be carried out by using a combination of known techniques such as techniques using ion exchange resins,

precipitation methods and so forth.

Brief Description of the Drawing

Fig. 1 is a graph showing inhibition by mecillinam for binding of PBP3 and penicillin G. The arrow indicates the minimum inhibitory concentration.

Best Mode for Carrying out the Invention

Hereafter, examples of the present invention will be explained.

Example 1: Detection of PBP of coryneform bacteria

Detection of PBP of coryneform bacteria was performed based on the aforementioned method of Spratt, *et al.*, and specifically as follows.

<1> Preparation of membrane fraction

PBP is considered to be a membrane protein. A membrane fraction of a coryneform bacterium was prepared as follows. A wild strain of coryneform bacteria, *Brevibacterium lactofermentum* ATCC 13869 strain, was inoculated to 20 ml of A medium (containing 10 g of polypeptone, 5 g of yeast extract, 5 g of NaCl and 5 g of glucose in 1 L of water), cultured at 30°C with shaking, and harvested when the absorption at 660 nm reached about 1.0. The cells were washed in 50 mM

sodium phosphate buffer, pH 7.0. Then, the buffer was added with glass beads and sonicated to disrupt the cells. After the disruption debris was removed by centrifugation, the buffer was subjected to ultracentrifugation at 100,000 x g for 30 minutes to collect a membrane fraction. The obtained fraction was washed with the same buffer, and the fraction finally suspended in 500 μl of the same buffer was used as the membrane fraction. Protein concentration of this membrane fraction was determined by using a protein quantification kit (produced by PIERCE, Micro BCA Protein assay kit), and found to be 4 mg/ml.

<2> Penicillin binding reaction

A volume of 30 μl of the prepared membrane fraction was added with 3 μl of ^{14}C-penicillin G (produced by Amersham) and allowed to react at 30°C for 10 minutes. Then, it was added with 2 μl of 15% sarcosyl (sodium N-lauroylsarcosine) and 45 mg/ml of penicillin G, and left for 20 minutes at room temperature. Then, the fraction was centrifuged at 13,000 rpm for 30 minutes at 20°C to obtain a soluble fraction. A sample of this fraction was subjected to SDS-PAGE using gel containing 10% of polyacrylamide, and the gel was fixed, dried and analyzed with an image analyzer BAS2000 produced by Fuji Photo Film Co., Ltd. As a result, three bands were detected at 110 kDa, 100

kDa and 60 kDa, and designated as PBP1, PBP2 and PBP3, respectively.

<3> Analysis using PBP binding inhibitor

Affinity between mecillinam, which is a derivative of penicillin G, and PBP was investigated. The affinity between the derivative and PBP was investigated by adding mecillinam at various concentrations before the aforementioned penicillin binding reaction, and measuring degree of inhibition of the binding with isotope (^{14}C) labeled penicillin G. As a result, only PBP3 was inhibited for binding with the labeled penicillin G by the addition of mecillinam. That is, it was clarified that mecillinam should selectively bind to PBP3 (Fig. 1).

Example 2: Glutamic acid production by coryneform bacterium induced by addition of mecillinam

The *Brevibacterium lactofermentum* ATCC 13869 strain was inoculated to the aforementioned A medium, cultured at 30°C for 2 hours with shaking, then added with 0.01 μM to 100 μM of mecillinam, and further cultured for 8 hours with shaking. Subsequently, glutamic acid concentration in the medium was measured by using Biotech Analyzer AS210 (produced by Asahi Chemical Industry Co., Ltd.) (Table 1).

Table 1

Concentration of added mecillinam (μM)	Concentration of L-glutamic acid (mg/L)
0	0
0.1	0
1.0	0
10	0
100	575

Since it was only PBP3 that bound to mecillinam under the condition where L-glutamic acid production was induced by addition of mecillinam, it was demonstrated that the glutamic acid production induced by the addition of penicillin or mecillinam was caused at least by inhibition of the function of PBP3.

Example 3: Cloning of PBP gene of *Brevibacterium lactofermentum* ATCC 13869

(1) Preparation of chromosomal DNA of *Brevibacterium lactofermentum* ATCC 13869

The *Brevibacterium lactofermentum* ATCC 13869 strain was cultured overnight in 10 ml of L medium (1% of polypeptone, 0.5% of yeast extract, 0.5 g of NaCl, 0.1% of glucose, pH 7.2), and harvested. The cells were washed with 50 mM Tris-HCl, 50 mM EDTA buffer (pH 8.0), and suspended in 800 μl of the same buffer.

To the aforementioned cell suspension, 40 μl of a 50 ml/ml lysozyme solution and 20 μl of a 10 mg/ml ribonuclease solution were added, and the mixture was incubated at 37°C for 1 hour. The mixture was added

with 20 μl of a 20% SDS solution, and incubated at 70°C for 1 hour. Then, the mixture was added with 24 μl of a 20 mg/ml proteinase K solution, incubated at 50°C for 1 hour, further added with 24 μl of the proteinase K solution, and incubated for 1 hour.

The cell lysate obtained as described above was added with an equal amount of phenol and stirred. The lysate was left at 4°C overnight, and then centrifuged to collect an aqueous layer. The aqueous layer was extracted with phenol/chloroform for 2 hours, and with chloroform/isoamyl alcohol for 30 minutes. The extraction was carried out by leaving the lysate after stirring for a predetermined period of time and centrifuging it to collect an aqueous layer. DNA was collected from the obtained extract by ethanol precipitation. The precipitation of DNA was dissolved in 300 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

(2) Preparation of chromosomal DNA library of *Brevibacterium lactofermentum* ATCC 13869

The chromosomal DNA of the *Brevibacterium lactofermentum* ATCC 13869 obtained as described above and a high copy number vector pHSG398 of *Escherichia coli* (Takara Shuzo) were digested by with a restriction enzyme HindIII (Takara Shuzo). These two of DNA were mixed in suitable amounts and ligated by using Takara

DNA Ligation Kit ver. 2 (Takara Shuzo) to construct a chromosomal DNA library of *Brevibacterium lactofermentum* ATCC 13869.

(3) Selection of PBP gene clone

A murE mutant strain TLK11 of *Escherichia coli* (murE temperature sensitive mutation, J. Bacteriol., 1972, 110:41-46) was transformed with the chromosomal DNA library of the *Brevibacterium lactofermentum* ATCC 13869 obtained as described above, and cultured at 42°C overnight on L agar medium (L medium containing 1.5% of agar) containing 20 μg/ml of chloramphenicol.

An emerged colony was inoculated to L liquid medium, and cultured, and plasmid was collected from the obtained cells. As a result, a HindIII fragment of about 5.3 kb was cloned in the plasmid. This plasmid was designated as pHSGH-H.

(4) Determination of nucleotide sequence of cloned DNA fragment

The aforementioned cloned fragment was subjected to the dideoxy reaction using Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) and determined for the nucleotide sequence by using a DNA sequencer DSQ-1000L (Shimadzu). The determined nucleotide sequence is shown as SEQ ID NO: 1.

Example 4: Construction of PBP gene-disrupted strain of *Brevibacterium lactofermentum*

A *Brevibacterium lactofermentum* strain with disrupted PBP gene on the chromosome was created by the homologous recombination using the temperature sensitive plasmid disclosed in Japanese Patent Laid-open No. 5-7491.

PCR was performed by using pHSGH-H as a template and oligonucleotides having the nucleotide sequences shown as SEQ ID NOS: 5 and 6 as primers to amplify a PBP gene fragment. The primer shown as SEQ ID NO: 5 contained a sequence corresponding to nucleotide numbers 31-50 of the nucleotide sequence shown as SEQ ID NO: 1, and the primer shown as SEQ ID NO: 6 contained a sequence corresponding to nucleotide numbers 2991-3014 of the nucleotide sequence shown as SEQ ID NO: 1, and each had an EcoRI recognition sequence in the 5' end sequence. The PCR reaction was carried out by using a commercially available PCR apparatus (DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo etc.), and TaqDNA Polymerase (Takara Shuzo) according to the manufacturer's protocol.

The obtained amplified fragment was treated with EcoRI, and inserted into the EcoRI site of pHSG299 (produced by Takara Shuzo) to produce pHSGE. Separately, the temperature sensitive plasmid for coryneform

bacteria, pHSC4, was digested with BamHI and KpnI to obtain a gene fragment containing a replication control region, and the obtained fragment was blunt-ended by using Blunting kit produced by Takara Shuzo, and inserted into the XbaI site of pHSGE using a XbaI linker (produced by Takara Shuzo) to produce pHSGX. Then, pHSGX was digested with BamHI and KpnI, blunt-ended by using Blunting kit produced by Takara Shuzo and allowed to cause self-ligation by using Takara DNA Ligation Kit ver. 2 (Takara Shuzo). The obtained plasmid pHSGXΔBK had an internal deletion of the PBP gene.

By using the plasmid pHSGXΔBK for PBP gene substitution obtained as described above, gene substitution between a deletion type PBP gene and a PBP gene on chromosomal DNA of *Brevibacterium lactofermentum* was performed by the double recombination technique. Specifically, it was attained as follows. The *Brevibacterium lactofermentum* ATCC 13869 was transformed with pHSGXΔBK by the electric pulse method (see Japanese Patent Laid-open No. 2-207791). The transformant was cultured at 25°C for 6 hours in M-CM2G liquid medium with shaking and inoculated on M-CM2G medium containing 25 μg/ml of kanamycin. A strain that formed a colony at 34°C was obtained.

In the obtained strain, the deletion type PBP gene was recombined with the PBP gene originally present on the chromosome, and the two fusion genes of the

chromosomal PBP gene and the deletion type PBP gene are inserted into the chromosome so that the other portions of the recombinant DNA (vector segment, temperature sensitive replication control region and drug resistance marker) should be present between the two fusion genes. Therefore, the transformant strain could grow at a high temperature because the normal PBP gene was dominant in this state.

Then, the transformant strain was cultured at 25°C, and a strain in which the PBP gene on the chromosome was replaced with the deletion type gene was selected from the strains that could grow at 25°C but could not grow at 34°C, and designated as ΔP3/p3 strain. In the ΔP3/p3 strain, the deletion type PBP gene was left on the chromosomal DNA, and a plasmid containing a normal PBP gene was present in the cell. It was confirmed if the PBP gene on the chromosome was replaced with the deletion type by determining the nucleotide sequence of the ligation region left behind the deleted region.

Industrial Applicability

According to the present invention, a gene coding for a penicillin binding protein (PBP gene) of a coryneform bacterium is provided. By using a coryneform bacterium of which PBP gene on the chromosome was disrupted with the gene, L-glutamic acid can be produced

in the presence of a significant amount of biotin without adding a biotin activity suppressor.